In silico approach towards finding inhibitory effect of phytochemicals on Trypanothione reductase in *Leishmania donovani*

A Thesis submitted in partial fulfilment of the requirements for the degree of

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In

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By

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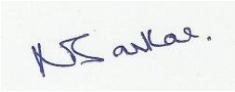
CERTIFICATE

This is to confirm that the Thesis entitled **"In silico approach towards finding inhibitory effect of phytochemicals on Trypanothione reductase in Leishmania donovani"** by **Ritika Chauhan (212BM2358)** submitted to the National Institute of Technology, Rourkela for the honour of Master of Technology in Biotechnology during the session 2012-2014 is a record of Bonafide research work did by her in the Department of Biotechnology and Medical Engineering under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.



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ABSTRACT

Problem statement: Leishmaniasis caused by *Leishmania* sp. is second most dangerous tropical disease without any specified therapeutic system available. Trypanothione reductase plays a key role in survival of the protozoa which can be used as target for Structure based Drug designing. Phytochemicals provides a less toxic and accessible source of chemotherapeutic agents. The study deals with discovery of one such agent against the targeted protein for further Anti-leishmanial drug discovery.

Method: An in-silico approach was executed in order to find out the structure of the targeted protein and prominent lead compound as it potential inhibitor.

Result: The structure of Trypanothione reductase was obtained and validated. It was found to be a compatible model suited for further investigation. The docking studies performed provided Curcumin as the potential chemotherapeutic agent giving a high binding energy of -7.9 kcal/mol. The analogues of curcumin presented higher binding energy than base compound. Further ADME/T tests suggested two analogues to be prevalent for further study with binding energy of -11.0 kcal/mol and -8.8 kcal/mol.

Keywords: Leishmaniasis, *Leishmania,* Trypanothione reductase, Phytochemicals, Curcumin, *in-silico*, Docking, Chemotherapeutic, ADME/T

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ABBREVIATIONS

TR/TPR	Trypanothione reductase
GR	Glutathione reductase
VL	Visceral Leishmaniasis
NADPH	Nicotinamide Adenine Dineucleotide phosphate
FAD	Flavin Adenine Dineucleotide
Leu	Leucine
Val	Valine
Ala	Alanine
Phe	Phenylalanine
Ile	Isoleucine
Tyr	Tyrosine
His	Histidine
Cys	Cystine
Pro	Proline
Gly	Glycine
Thr	Threonine
Met	Methionine
Arg	Arginine

CHAPTER 1

INTRODUCTION

1.1 Introduction

1.1.1 Leishmaniasis

Trypanosomatids are the cause of several fatal forms of tropical human diseases including Leishmaniasis. There are over twenty different species of *Leishmania* causing the disease. Leishmaniasis is the wide variety of pathological manifestations that impact differently on health and that differ in their severity depending on its interaction along with HIV and other medical complications [1]. Several species of flagellated parasite of the order Kinetoplastidae, genus *Leishmania,* are responsible for this group of zoonotic infections of men and certain animals which is transmitted fly of the genera *Phlebotomus* and *Lutzomyia* bite the person. Once inoculation into the skin takes place, the parasites rapidlyllocate to the phagolysosomes of the mononuclearlphagocyte system. Human infection can be caused by several species of *Leishmania* which are included in four complexes: (1) *tropica* (*L. tropica, L. major, L. minor,* and *L. aethiopica*); (2) *mexicana* (*L. mexicana, L. amazonensis, L. pifanoi,* and *L. venezuelensis*); (3) *braziliensis* or *viannia* (*L. brasiliensis, L guyanensis, L. panamensis,* and *L. peruviana*); and (4) *donovani* (*L. donovani, L. infantum, L. chagasi, L. sinesis,* and *L. nilotica*). The important clinical syndromes of Leishmaniasis include:

- Cutaneous leishmaniasis;
- Muco-cutaneous leishmaniasis (also referred to as espundia);
- Visceral leishmaniasis (called as VL; kala-azar); and
- Post-kala-azar dermal leishmaniasis (PKDL).

VL is instigated by the *Leishmania donovani* complex — *L. donovani* causing in the subcontinent of India and East Africa and *L. infantum* in Europe, Latin America and North Africa is a systemic disease that is lethal if left untreated. Every year there are around 500,000 new cases of VL and over 50,000 deaths from the disease, making it the second most fatal parasitic disease after Malaria [2].

Various drugs have been tested and clinically tried for the particular disease but no efficient result has been found as there are always some side effects related to the treatment. This condition makes it more complex to design a proper drug system. The vigorous research is going on in various places but the strain which is the main causative agent of the VL in India is yet under researched due to its incomplete information.

1.2 Leishmaniasis and Trypanothione reductase

Trypanothione reductase, an NADPH-dependent flavoprotein which is found specifically in protozoan parasites from the genera *Trypanosoma* and *Leishmania* is a associate of the disulfide oxidoreductase family of enzymes. The NADPH and the thiol-based redox system are connected by this key enzyme. Inhibition studies have revealed the importance of TPR in the survival of the parasite. Therefore, it is a rational target for the development of newtdrugs by coherent inhibitor design [3].

1.3 Phytochemicals

The medicinal plants possess their property of healing as well as of curing of human diseases because of the presence of phytochemical constituents. The term "phytochemicals" refers to a extensive variety of compounds produced by plants, but it is generally used to designate those compounds that possibly have an impact on human health. Instead of identification of about thousands of phytochemicals, only a small fraction has been studied closely. Phytochemicals are primary and secondary compounds [22]. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoid, alkaloids and phenolic compounds. Phytochemicals have proven themselves to be potential chemopreventive and chemotherapeutic agents [25].

1.3.1 Curcumin

Curcumin is one of three curcuminoids found in *Curcuma longa* (turmeric), the natural phenol is responsible for the yellow color of turmeric. Curcumin is a proven agent, responsible for most the biological functions of *C.longa*. The effect of curcumin is being tested through clinical trials in humans on various diseases, including multiple myeloma, cancer, myelodysplastic syndromes, psoriasis, arthritis, major depressive disorder and Alzheimer's disease [27]. The efficacy of Curcumin and its analogues against Leishmaniasis have been tested for further drug designing. The Pharmacological grade Curcumin acts as an Anti-inflammation, Non-Steroidal, Antineoplastic, Coloring agent and an Enzyme inhibitor [28].

1.4 Bioinformatics and Drug discovery

The multi-step process of drug discovery starts with target and lead discovery, followed by lead optimization and pre-clinical *in vitro* and *in vivo* studies to define if such compounds fulfill a number of pre-set standards for commencing clinical improvement. The advent of advanced genomics, proteomics and bioinformatics and proficient technologies as High Throughput Screening (HTS), virtual screening, *de novo* synthesis, in silico ADMET test and structure based drug designing revolutionizes this simple method.

The advanced computational methods, internet sources and ease of their use form the central foundation of Structure-based drug designing. Huge amounts of data are being accessed and used efficiently for the purpose of drug discovery by means of high-performance computing analysis, data management software and internet. The computational techniques increase the chance of success in drug discovery and in the design of desirable lead compounds. Computation plays a major role in drug designing and discovery at the following 3 stages:

- Virtual screening and *de-novo* designing
- ADME/T prediction
- Methods for studying protein-ligand binding

1.5 Objective

- To identify appropriate target protein for drug discovery against Leishmaniasis.
- To predict and validate the structure of the enzyme Trypanothione Reductase.
- To compare the structure with its human analogue (glutathione reductase) to suggest its specific action.
- To find out potential lead compound (phytochemical) for inhibition of the target enzyme, Trypanothione reductase.
- To design novel Phytochemical (Curcumin) analogues as Trypanothione inhibitors.
- To analyze inhibition action of the analogues through protein ligand interaction using AUTODOCK.
- To perform ADME/T test on the analogues.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1 Leishmaniasis

A group of diseases ranging from self-curing lesions to gross disfigurations and potentially deadly visceral disease, by the name Leishmaniasis, are caused by protozoan parasites that are transferred by sandflies [1]. The Leishmania genus parasites present enormous differences in disease tropism, due to their diverse biological, clinical, and epidemiological nature. *Leishmania major* and other species cause the mildest form of cutaneous leishmaniasis; largely limited to lesions around the area of a sandfly bite—though a diffuse form can also occur. The destruction of nasopharyngeal tissue by parasites *L. braziliensis* causes disfiguring muco-cutaneous leishmaniasis. Visceral leishmaniasis is caused by the parasites of *L. donovani* species complex that can spread to internal organs and cause death [2,3].

2.2 Clinical presentation of Visceral Leishmaniasis

The general infection of liver, spleen and bone marrow results in VL which can affect both new and old world species [3]. The characterizing symptoms caused by prolonged infection constitute the pentad of fever, weight loss, hepatosplenomegaly, pancytopenia, and hypergammaglobulinemia. Patients may suffer with night sweats, weakness, and anorexia. Characteristic skin hyperpigmentation is caused by melanocyte stimulation and xerosis [4]. After infection the incubation period varies depending on the age and immune status of the patient and the species of *Leishmania*. Non-treatment leads to frequent death from immunosuppression and secondary infections [5].

The symptoms of VL are generic in various endemic areas, except some specific characteristic cases like enlarged lymph nodes, frequent in Sudanese VL patients, rarely found in VL patients in India. Hyper-pigmentation, an uncommon symptom and a plausible reason for the name ‘kala-azar’, has only been observe in VL patients from the Indian sub-continent, was a feature of prolonged illness in an era when nominal treatment was not available [3,6]. Clinical image at the initiation of diagnosis is hindered in VL due to symptoms and marks of bacterial co-infections such as pneumonia, diarrhoea or tuberculosis, which often tend to continue for several weeks before patients either seek medical care or die from bacterial co-infections, massive bleeding or severe anaemia [4].

2.3 Pathogenesis of Leishmaniasis

The two distinct forms of the lifecycle of *L. donovani*:

- Promastigote flagellar in the gut of the arthropod vector and
- Amastigote form, which grows intracellularly in the mammalian host [4].

The disease transmission occurs only by female phlebotomine sandflies, by inoculation of the promastigote form into the skin. The internalization of the parasites takes place by dendritic cells and macrophages in the dermis and it is transformed in amastigotes after losing their flagella [11]. They form a complex parasite–host interaction in phagolysosomes for their multiplication and survival. Reticulo-endothelial system is compromised due to infection of other monocytes and macrophages by the dissemination of the parasite through the lymphatic and vascular systems resulting in infiltration of the bone marrow, hepatosplenomegaly and sometimes enlarged lymph nodes (lymphadenopathy) [5, 9].

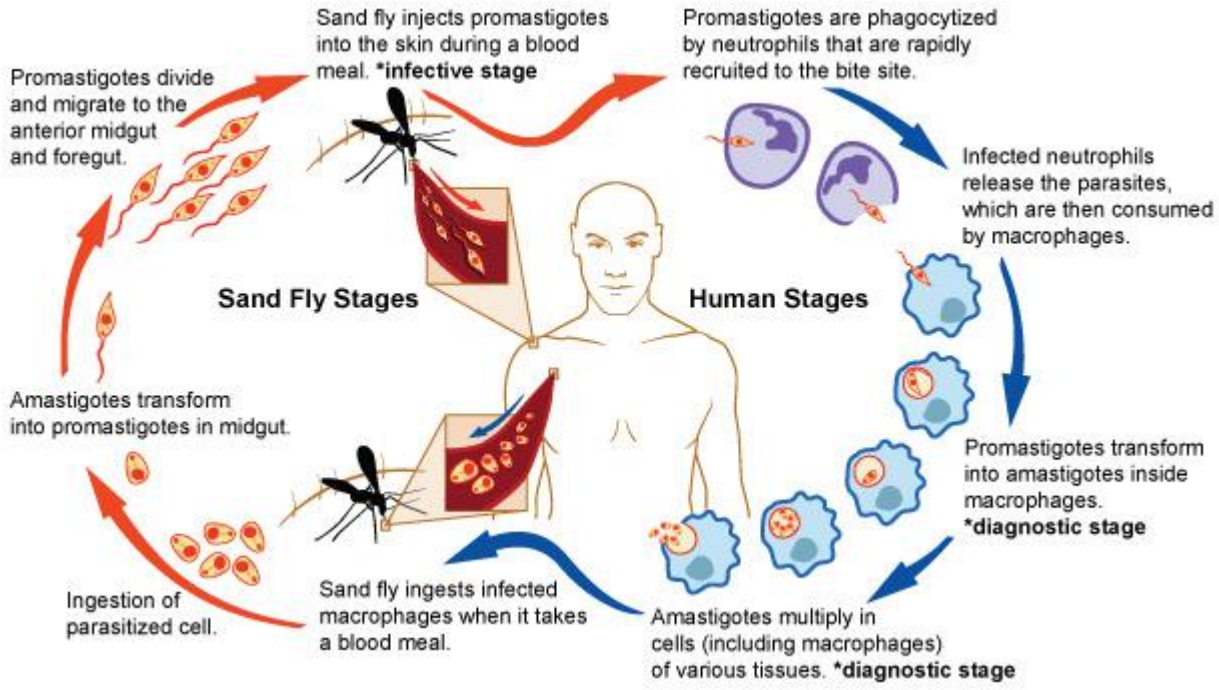


Fig 2.1: Lifecycle of *Leishmania donovani* [4]

The infection is controlled by the host specific cell-mediated immune (CMI) response. The incapability to control *L.donovani* infection in the VL prone subjects is related with a profound T-cell unresponsiveness to its antigens and the production of interleukin 10 [17].

2.4 Trypanothione reductase in *Leishmania* species

Development of a rational approach to the design of new anti-leishmanials released new aspects in leishmania biochemistry which can be explored. A targeted study is the intracellular dithiol trypanothione [N^1,N^8-bis(glutathionyl)spermidine], exclusive to all kinetoplastida including the parasites responsible for Chagas' disease *(Trypanosoma cruzi)* and African trypanosomiasis *(Trypanosoma brucei* spp) and the *Leishmania* spp [21]. Many of the anti-oxidant and other protective functions ascribed to glutathione in other organisms including mammals are executed by Trypanothione $T(SH)_2$, being a major low-molecular-mass thiol in these parasites. In comparison to GSH, $T(SH)_2$ is a much more efficient protector against $^{\bullet}NO$ [6,18]. This reliability of Trypanosomatids on T $(SH)_2$ assists them in controlling the redox states on exposure to higher $^{\bullet}NO$ levels in the mammalian host. The intracellular level of dihydro trypanothione is maintained by trypanothione reductase (TR) and subsequently results in the maintenance of the reducing environment [19]. TR down-regulation studies show its vitality in the parasite's physiology, in the case of Leishmania. Also, TR down-regulation weakens infectivity and survival in cytokine-stimulated macrophages. TRs are structurally analogous to GRs and are members of the family of NADPH-dependent flavoprotein oxidoreductase. The reduction of $T(S)_2$ [7,10] by TR is aided by the use of NADPH as an e^- donor. TRs possess distinct substrate specifities in comparison to human GR. Hence; TR makes an optimum target for antiparasitic drugs. Consequently, humongous amounts of efforts are being invested in the pursuit for selective TR inhibitors in researching for the treatment of trypanosomiasis and leishmaniasis [15].

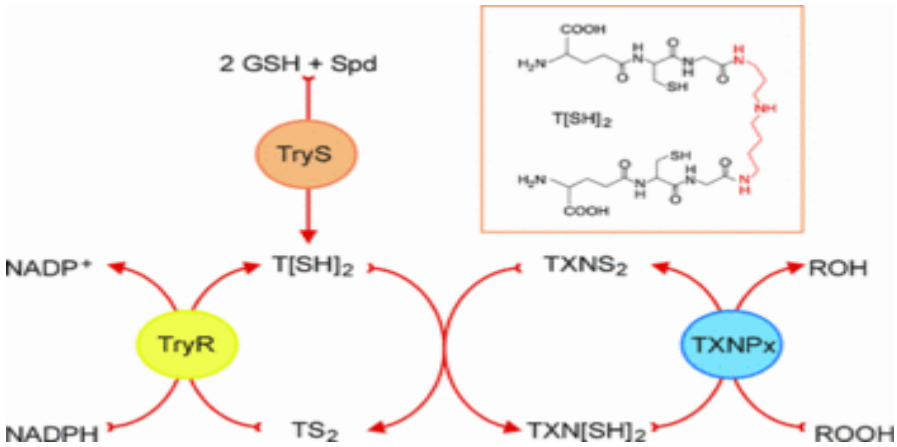


Fig 2.2 Action of Trypanothione reductase [6]

2.5 Therapeutic approach

The inhibition of disease at initial stage suggests the possibility of generation of vaccine against Leishmania but it has not yet been possible. There are limited drugs available for the treatment of VL and have shown various shortcomings [8]. Pentavalent antimony instead of being used as effective drug has become outdated because of development of resistance in some parts of the World. The drugs which are or have been used against Leishmania have shown negative outcomes at some point [1, 9]. The list of drugs is given below:

2.5.1 First Line Drugs:

- Sodium stibogluconate (Pentostam, SSG); meglumine antimoniate (Glucantime)
- Amphotericin B (Fungizone)
- Liposomal amphotericin B (AmBisome)
- Pentamidine

2.5.2 Clinical trials:

- Miltefosine (oral, Phase IV; registered in India) [16]
- Paromomycin (Phase III)
- Sitamaqine (oral, Phase II)
- Other amphotericin B formulations [7, 12, 13]

S.no	Drug	Limitations
1	Pentavalent antimonials	Variable efficacy, Resistance development
2	Amphotericin B	Adverse effects, High toxicity
3	Pentamidine	Low efficacy, High toxicity
4	Miltefosine	Renal toxicity, Teratogenicity
5	Paromomycin	Ototoxicity, Liver problems

Table 2.1 Limitations of prevalent drugs

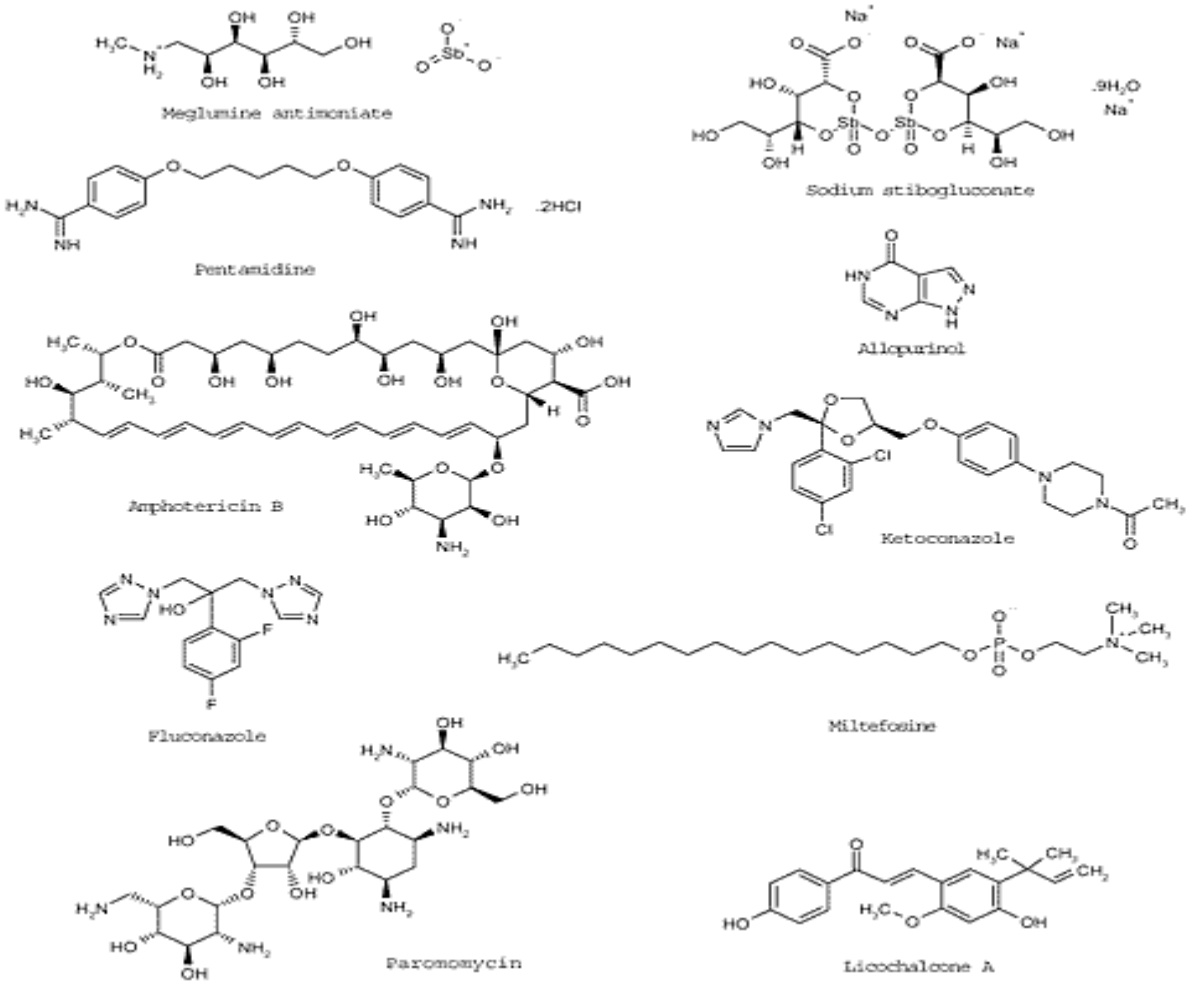


Fig 2.3: Structures of some common drugs in clinical trial [2]

2.6 Phytochemicals

The word 'phyto-' is Greek for plant. Bioactive, non-nutrient plant compounds obtained from in fruits, vegetables, grains, and other plant foods are termed as Phytochemicals. They, when altered to be edible, reduce the threat of major chronic diseases [20]. Their presence confers with resistance against pathogens, be it bacteria, fungi or protozoa. These bioactive components show remarkable antimicrobial effects *in vitro* leading to anti-pathogenicity of plants. In locales where commercial drugs are unavailable and/or are too expensive, and an abundance of medicinal herbs is evident, like Africa treatment of infections is often resorted with the local and indigenous plants [22]. Infectious pathologies are being treated by employment of plant parts and their components like roots, leaves, stem barks, flowers or combinations, essential oils in the urinary tract, respiratory system, biliary and gastro-intestinal systems along with the skin. A variety of phytochemicals, with antibacterial

activity, exist in plants. Plant materials are colossal and ubiquitous reservoir of the polyphenolic phytochemicals.

The side effects, generally caused by synthetic chemicals, are almost never observed in the plants, being the natural reservoirs of medicinal agents [23]. Herbal medicine is acceptable to be the main channel of medication for a large proportion of world population, primarily in the developing and third-world countries for primary health care, owing to better cultural acceptability and better compatibility with the human metabolism, and fewer side-effects. The abuse of synthetic drugs with impurities results in higher frequency of adverse drug reactions, motivating the researchers to retrace to natural substances for safer remedies [24]. The WHO published standards for herbal safety to minimize adulteration and abuse to avoid caused by various vernacular names due to widespread locations of remedial plants [12, 25]. The uses of the phytochemical agents in traditional medicine led to isolation of numerous modern drugs from natural sources. Evolution of a generalized defense mechanism against low levels of phytochemicals in human enables consumption of many different plant species containing variable levels of natural pesticides (carcinogens) without consequent side effects or ill health, acts in the favor of phytochemicals, among an projected 10,000 secondary products (natural pesticides) [30].

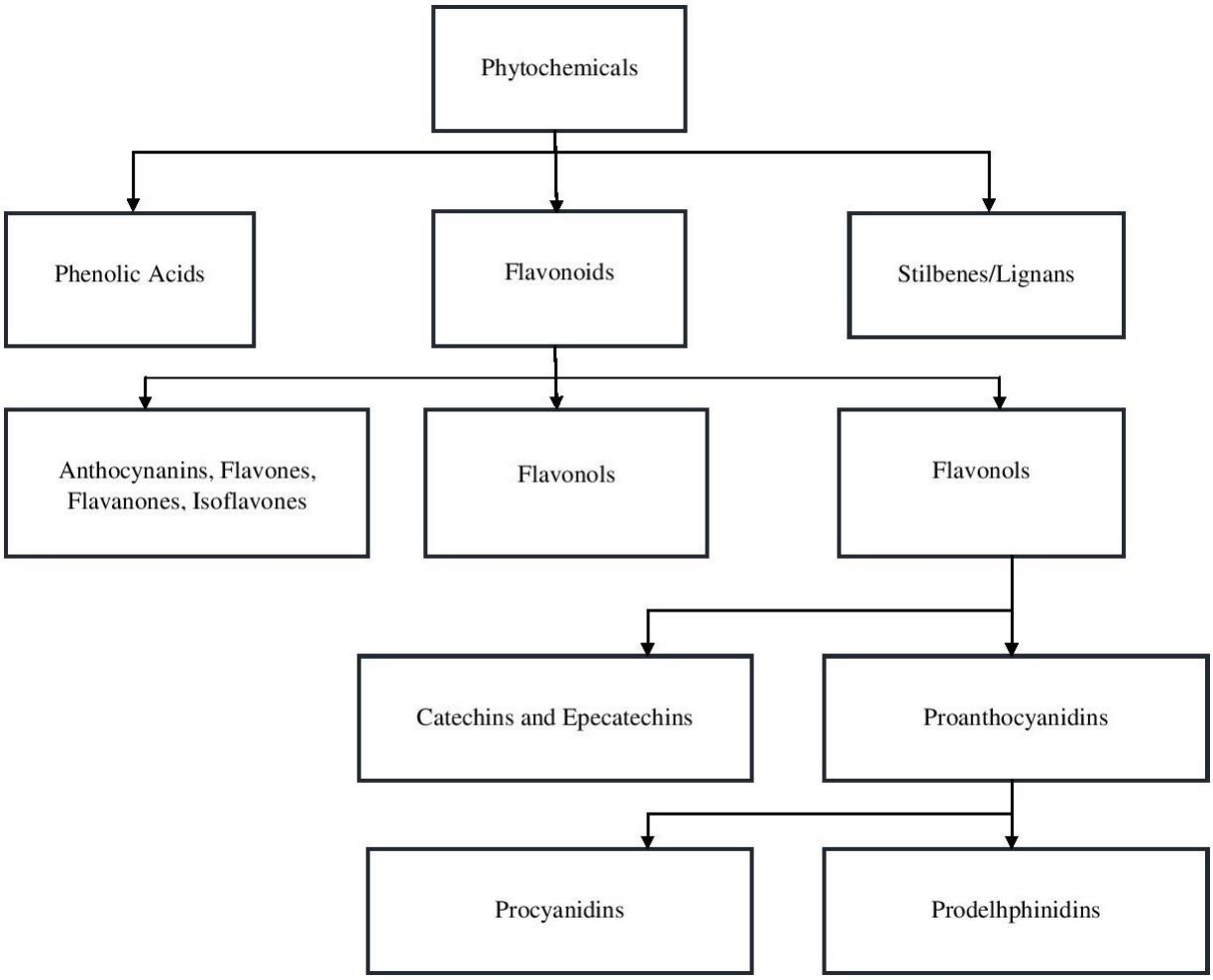


Fig 2.4: Chart showing distribution of Phytochemicals in Nature

Synthesis of Phenolics in plants is obtained from phenylalanine, resulting from the action of phenylalanine ammonia lyase (PAL). Plant phenolics play a vital part in the plant's defense mechanism against pathogens and herbivore predators, and thus, as a derivative, are used in limiting the human pathogenic infections [26].

Alkaloids, a class of natural, nitrogen-based, organic compounds, have diverse and important physiological effects on humans and other animals. Prominent examples include morphine, strychnine, quinine, ephedrine, and nicotine [25, 29].

Flavonoids, being the most diverse group of phytochemicals may prove to be an important phytochemical group contributing to the reduced mortality rates observed in people who consume high levels of plant-based foods [25].

A multitude of molecular targets can be targeted with various Phytochemicals such as epigallocatechin-3-gallate (EGCG) from green tea, curcumin from turmeric, and resveratrol from red wine. Definitive mechanisms of action for phytochemicals are not available despite extensive research, owing to these characteristics [30].

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Databases, Softwares and Tools used

3.1.1 UNIPROT

UNIPROT (Universal Protein Resource) is a inclusive freely accessible high quality database of protein sequences and their functional information. It provides 4 core databases:

i. UniProtKB- Protein database partially curated by experts
ii. UniParc- Protein comprehensive and non-redundant database
iii. UniRef- 3 databases of clustered sets of proteins from UniProtKB and UniParc
iv. UniMes- Database of Metagenomic and Environmental data

3.1.2 MODELLER 9.12

Modeller assists in homology or comparative 3D structure protein modeling. Spatial restraints consummation is implemented in protein structure modeling. It takes an alignment of the sequence to be modeled with known related structure and calculates a modelicontaining all non-hydrogen atoms [31, 32].

3.1.3 UCSF CHIMERA

UCSF Chimera is a highly extensible program for interactive visualization and analysis of molecular structures and related data, including sequence alignments, docking results, density maps, supramolecular assemblies, trajectories, and conformational ensembles. High quality images and movies can be generated. It is a freeware for academic purpose [33].

3.1.4 SAVes

SAVes (Structural Analysis and Verification Server) validates the modeled structure. It provides Ramachandram Plot analysis, PROCHECK analysis and ERRAT score. All of these parameters verify the structural stability and provides the refinement of the structure [34, 35].

3.1.5 CASTp

CASTp (Computed Atlas of Surface Topography of Proteins) istan online web server for prediction of binding sites and active sites of proteins and DNAs by determining the structural pockets and cavities in the protein [36].

3.1.6 RCSB PDB

It is a collection of 3D structures of large biomolecules such as proteins and nucleic acids, obtained by X-ray Crystallography and NMR spectroscopy. It’s a key resource in the area of Structural Biology.

3.1.7 LIGPLOT+

The software used for automatic generation of schematic diagram of protein-protein interaction and protein-ligand interaction.

DIMPLOT: Protein-protein interaction

LIGPLOT: Protein-ligand interaction [37]

3.1.8 PUBCHEM AND CHEMSPIDER

These are the free structural databases of chemical compounds. They provide the structural, functional and bioactivity information of the small molecules.

3.1.9 OPEN BABEL GUI

Open Babel is a chemical toolbox designed to communicate various languages of chemical data. It's an open, collective project which allows anyone to search, convert, analyze, or store data from molecular modeling, biochemistry, solid-state materials, chemistry, or related areas [38].

3.1.10 PRODRG

PRODRG takes a description of a small molecule (as PDB coordinates / MDL Molfile / SYBYL Mol2 file / text drawing) to generate energy-minimized coordinates various formats. This energy minimized molecule leads to better protein-ligand interaction and Docking results.

3.1.11 AUTODOCK 4.2

It is a suite of automated docking tools. It provides the prediction of binding of small molecules, such as substrates or drug candidates to a receptor of known 3D structure. It provides one of the best docking results and is a freeware for academic purpose. The procedure of Autodocking includes following steps:

1. **Preparation of Protein target**

- File -> Read molecule (Opens a file browser, Select target.pdb and click on Open)
- Edit -> Hydrogen -> Add Polar only
- Edit -> charges -> Add kollmann charges
- Edit -> atoms -> Assign AD4 type
- File -> save-> write pdbqt

2. **Preparation of Ligand File**

- Ligand -> Input Molecule -> Read Molecule (Choose ligand.pdb and Click on Open)
- Ligand -> torsion tree -> choose root
- Ligand -> torsion tree -> detect root
- Ligand -> output -> save as .pdbqt

3. **Preparing the macromolecule file.**

- Grid -> Macromolecule -> Choose Macromolecule
- Choose target
- Grid -> Set Map Types -> Choose Ligand -> select Ligand
- Grid -> Grid Box
- Opens the Grid Options
- Adjust the number of points in each dimension to 60.
- The spacing between grid points can be adjusted & the default value is 0.375 Å between grid points.
- Set the *x* center, *y* center and *z* center entries. This will center the grid box on the active site.
- Close this widget by clicking File ➞ Close saving current.
- Grid -> output -> save as .gpf

4. **Autogrid generation**

- Run -> Run AutoGrid (Opens the Run AutoGrid widget)

- Program Pathname: entry specifies the location of the autogrid3 executable.
- Parameter Filename: entry specifies the gpf file
- Log Filename: entry specifies the log file. Selecting a gpf creates a possible related name for the glg.
- Launch

5. Preparing the docking parameter file

- Docking -> Macromolecule -> Set Rigid Filename
- Docking -> Set Ligand Parameters -> Choose Ligand Click Select Ligand
- Docking -> Set Search Parameters -> Genetic Algorithm Parameters
- Docking -> output -> Lamarckian Genetic Algorithm (LGA)
- Save as .dpf file

6. Starting AutoDock

- Run -> Run AutoDock (Opens the Run AutoDock widget)
- Program Pathname: entry specifies the location of the autodock4 executable.
- Parameter Filename: entry specifies the dpf file.
- Log Filename: entry specifies the log file. Selecting a dpf creates a possible related name for the dlg.
- Launch

7. Saving results

- Open .dlg file
- Search for RMSD
- Copy the best RMSD calculation in target.pdb
- Save as result.pdb
- Observe using UCSF CHIMERA [39, 40]

3.1.12 MEDCHEM DESIGNER

It is a tool combining molecule drawing feature with basic ADME predictions. It can be used to design new molecules which can be saved in .sdf, .mol or smiles format and can then be used for further purpose.

3.1.13 OSIRIS Property Explorer

It is used to draw chemical structure and calculation of various drug relevant properties. It provides following properties:

1. Toxicity Risk assessment: The OSIRIS server searches for potential toxicity risks of the valid chemical structure provided on 4 major grounds: Mutagenecity, Tumorogenicity, Irritant, Reproductive impairment. Risk alert classify the structure as harmful according to the specified risk category. The compounds can be categorized as having low risk, medium risk and high risk.
2. cLogP: LogP is the logarithm of partition coefficient between n-octanol and water log of a compound which is a measure of hydrophilicity of compounds. High LogP value causes poor absorption or permeation specifying low hydrophilicity. For proper absorption of drug logP value must not be greater than 5.0
3. Solubility: It affects absorption and distribution characteristics of a compound. Low solubility leads to bad absorption and should be avoided. LogS value is unit stripped logarithm of solubility measure in mol/l. Efficient drugs have the estimated logS value greater than -4.0.
4. Molecular weight: Optimization of drug increases in accordance to the molecular weight of the compound but after a limit it tends to decreased bioactivity. Thus, MW of a compound has to be kept optimum. It is usually preferred to be lower than 500.
5. Drug likeness: The equation below sums up the score values present in the molecule under investigation and leads to calculation of drug likeness:

$$\mathbf{d= \Sigma v_i/\sqrt{n}}$$

   A positive value indicates presence of predominant fragments in commercial drugs.
6. Drug score: It is the combination of drug likeness, clogP, logS, MW and toxicity risks in one handy value to judge compound's overall potential to ualify for a drug. Its equation sums up as:

$$\mathbf{ds= \Pi\ (1/2 + ½\ s_i)\ .\ \Pi t_i}$$

ds= drug score

s_i= contribution calculated directly from clogP, logS, MW and drug likeness

t_i= contribution taken from 4 toxicity risk types

3.2 PROTOCOL

3.2.1 Modeling of Enzyme structure

Retrieval of amino acid sequence of Trypanothione reductase from UNIPROT

3D structure of enzyme was predicted using MODELLER 9.12 software

Energy minimization of structure was done using online server NOMAD-Ref

The structure validation was done using online SAVES server

Prediction of Active site was done using CASTp

Retrieval of 3D structure of Glutathione reductase from PDB

Superimposition of both enzymes using CHIMERA

LIGPLOT analysis for enzymes for FAD binding site

3.2.2 Inhibition study

Retrieval of phytochemicals from Pubchem and CHEMSPIDER database

Open Babel GUI was used to convert .sdf and .mol files to .pdb format

Energy minimization was done using PRODRG SERVER

Docking of TPR was done using AUTODOCK 4.2

Specific phytochemical was selected based on Binding affinity

3.2.3 Analogue study

Analogues of the selected compound were prepared using MEDCHEM DESIGNER

Open Babel was used to convert saved .mol format to .pdb format

Energy minimization was done using Prodrg server

Docking of analogues was done using AUTODOCK 4.2

Osiris server was used to test ADME/T

Retrieval of the residues interacting using LIGPLOT

3.3 Methodology

3.3.1 Selection of Enzyme

The enzyme was selected on the basis of its functioning in the pathogen. As the enzyme Trypanothione reductase plays a significant role in maintaining the Leishmania donovani, it was taken as the target. The 3D structure of the trypanothione reductase is not available so the amino acid sequence was taken from the UNIPROT database ID P39050 (http://www.uniprot.org/uniprot/P39050).

i. UNIPROT webpage was browsed.

ii. Protein Knowledgebase was searched for Trypanothione reductase in Leishmania donovani.

iii. Accession number P39050 was selected.

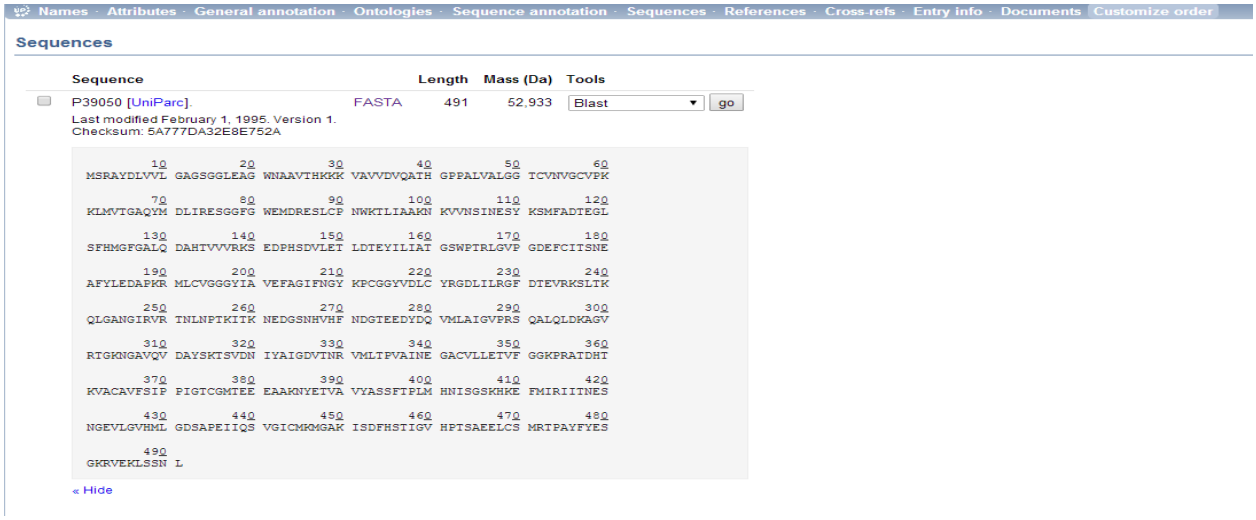


Fig 3.1: Snap shot showing protein sequence of TPR as obtained from UNIPROTKB

3.3.2 Homology modeling of the enzyme

The amino acid sequence was used as input for finding the homologous protein sequences with 3D structure using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program against PDB database in NCBI. The amino acid sequence of trypanothione reductase was aligned with the identified homologous proteins using Clustal X for understanding the conservation of amino acids throughout the protein family. The three dimensional structure of trypanothione reductase from the cytoplasm of *L.donovani* was predicted using trypanothione reductase from *L.infantum* (PDB ID: 2JK6) with Modeller 9.12 software (http://www.salilab.org/modeller) using sequence alignment and structural coordinates of the

template as input. The initial hundred models generated for Trypanothione reductase were prioritized on the basis of MOLPDF, discrete optimized protein energy (DOPE) and GA341 score.

i. Structure related to TPR was searched.
ii. Template was selected.
iii. TPR was aligned with the template; here Trypanothione reductase of Leishmania infantum.
iv. Model was built.
v. Model was evaluated.

Energy minimization of protein is done in order to release internal constraints which lead to developing of a more stable structure. This was done using online NOMAD-Ref server (Normal Mode Analysis, Deformation and Refinement). The structure was submitted to the server and the minimized structure was obtained.

The final model obtained with reasonable statistics (MOLPDF and DOPE) was validated in SAVES server (http://nihserver.mbi.ucla.edu/SAVES/). The final model was analyzed with PROCHECK and ERRAT plot and visually inspected with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

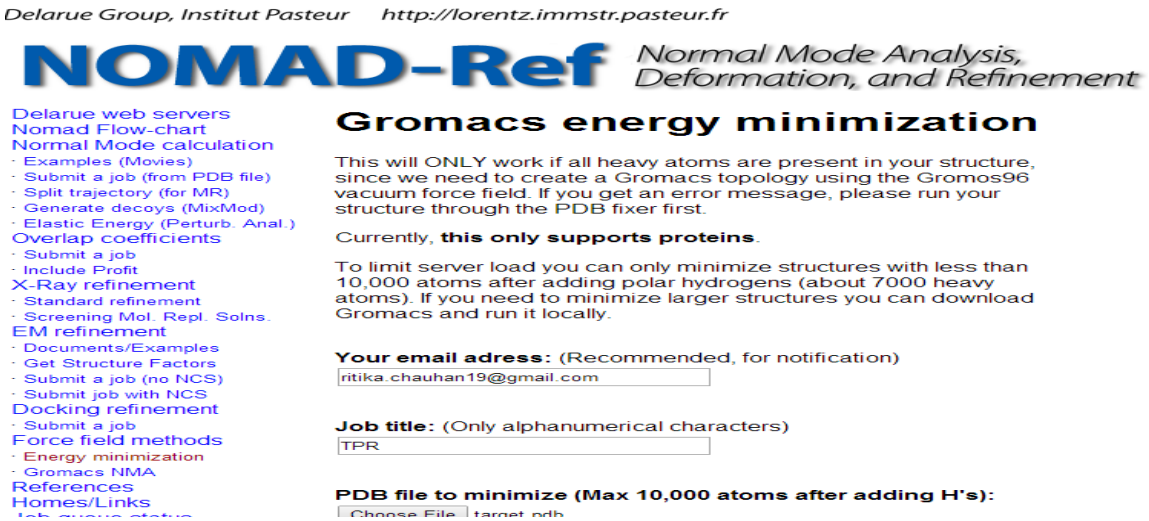


Fig 3.2: Snap shot showing protein submission for energy minimization in NOMAD-Ref

3.3.3 Prediction of Active Site

The small port of enzyme meant for substrate binding can be predicted using various tools available. This provides the pocket for the binding of the substrate and plays a vital role in action of the enzyme. The active site prediction was done using CASTp server It uses the weighted Delaunay triangulation and the alpha complex for shape measurements. It provides

i. Identification and measurements of surface accessible pockets.
ii. Interior inaccessible cavities, for proteins and other molecules.
iii. Analytical measurement of the area and volume of each pocket and cavity, both in solvent accessible surface (SA, Richards' surface) and molecular surface (MS, Connolly's surface).
iv. Measurement of the number of mouth openings, area of the openings and circumference of mouth lips, in both SA and MS surfaces for each pocket.

Retrieval of 3D structure of Glutathione reductase

The PDB (Protein Data Bank) is the universal store of Structural data of Biological macromolecules. In it the structures of macromolecules assessed by X-ray Crystallography and NMR are available. Glutathione reductase is human analogue of TPR. Its 3D structure was obtained from PDB (http://www.rcsb.org/pdb). In the process:

i. The mentioned url was searched.
ii. The query was given in the search.
iii. Various search options were obtained but the structure id 3GRS was selected.

This structure was superimposed on the TPR structure to observe the difference between the structure and their potential ligand binding sites.

3.3.4 Retrieval of phytochemical structures

The structures were obtained from Pubchem or CHEMSPIDER which are the databases of chemical compounds and micromolecules. The 3D structures downloaded in .sdf or .mol format were converted to .pdb format using Open Babel GUI and were observed using CHIMERA software. For few ligands the 3D structure was not available. In that case the 2D SMILES format was input online in CORINA (http://www.molecular-networks.com/online_demos/corina_demo) to obtain the 3D structure. The energy minimization of the ligands was done using Ligandscout.

3.3.5 Docking

Docking for the study of binding affinity was done using AUTODOCK 4.2 which provided the binding affinity of TPR with various phytochemicals. The 3D structure visualization was done using UCSF Chimera. Based on these docking values and further specific activity of the phytochemical Curcumin was selected for further study.

3.3.6 Analogue design

MEDCHEM DESIGNER was used to vary the structure

i. Medchem window was opened.
ii. Ligand .mol format was retrieved.
iii. Change in functional groups by addition or deletion was done.
iv. The file was saved in .mol format.
v. Open Babel was used to convert .mol to .pdb.
vi. Energy minimization was done using the PRODRG server.

3.3.7 Docking of Analogues

Docking of the energy minimized analogues of Curcumin and TPR was again done using Autodock 4.2.

3.3.8 ADME/T test

ADME/T test was done using MOLSOFT (https://molsoft.com/mprop/) and OSIRIS server (http://www.organic-chemistry.org/prog/peo/).

i. Open Babel was used to convert .pdb or .mol or .sdf format to SMILES.
ii. These smiles were copied in the window provided (in case of Osiris it has to be typed or CAS no. of available compound can be typed and the structure can be varied accordingly in the window.)
iii. The ADME/T and drug likeness score was obtained.

3.3.9 Ligplot analysis

It was performed using Ligplot+ which is a free ware.

i. The Ligplot window was opened.
ii. Protein-ligand complex in .pdb format was selected.
iii. Ligand was selected for the interaction study and the plot window was opened.
iv. The plot was saved.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Homology Modeling

The sequence analysis of Trypanothione reductase *L.donovani* showed maximum homology with the Trypanothione reductase of *L.infantum* (PDB ID: 2JK6) which was showing high similarity (>85%) with the structure. Thus, this was taken as the template for the modeling of the target enzyme. The 3D structure of the Trypanothione reductase enzyme was generated using MODELLER tool.

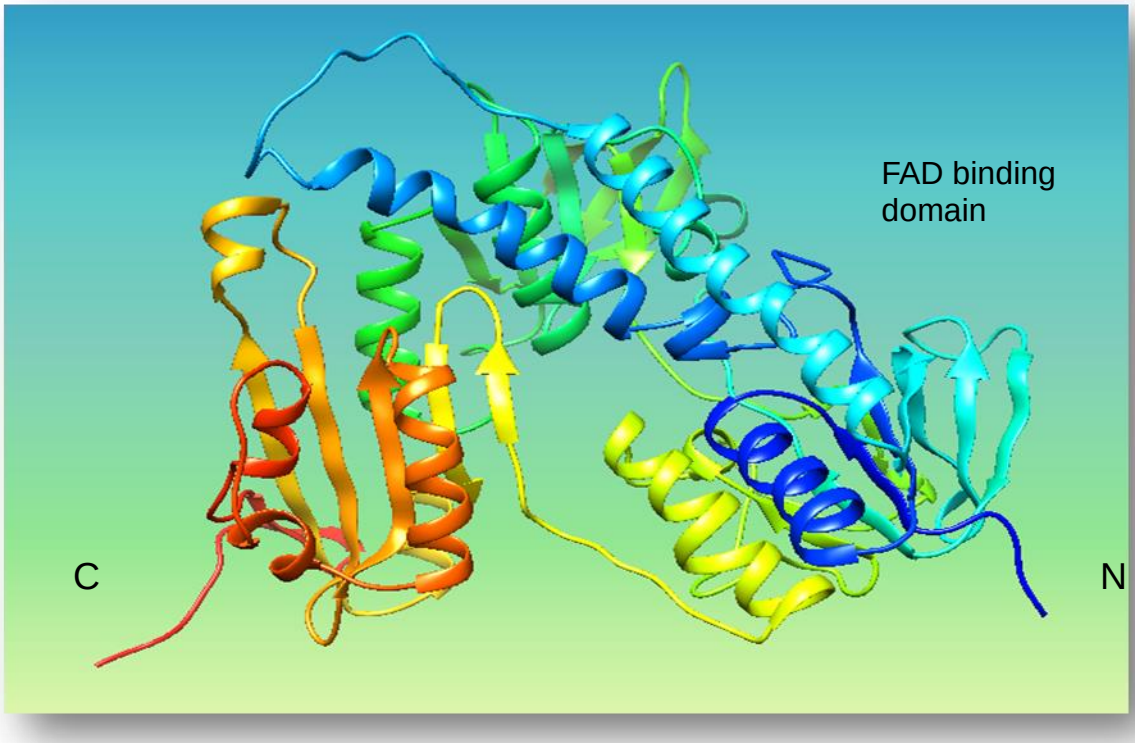


Fig 4.1: Structure obtained by homology modeling as viewed in Chimera

The protein ribbon is rainbow-colored from blue at the N-terminus to red at the C-terminus using CHIMERA. The spiral structures are the α helices and the arrows represent the β sheets. The thread in between are coils and turns.

4.1.2 Structure Validation

The quality of the modeled structure was validated by Ramachandran plot using PROCHECK and ERRAT plot. Ramachandran Plot is a plot of torsional angles: ψ (psi) the C-Cα torsional angle against φ (phi) the N-Cα torsional angle of the residues (amino acids) in a peptide/protein. A Ramachandran Plot specifies the torsional angles in a protein which are allowed and thus provides an insight to the conformations of the amino acids and geometry of the protein. The PROCHECK analysis showed that 93.1% structure was in most favored region, 6% was in additional allowed region, 0.2% in generously allowed region and 0.7% (3 residues LEU45, VAL287, ALA363) in disallowed region. The overall quality factor of the model from ERRAT, a protein structure verification algorithm, was 90.24% which stipulate that the structure is having low steric hindrance and is well in the range of good statistics. This plays a key role in determining the structural stability of the proteins and its refinement, obtained through crystallographic model building.

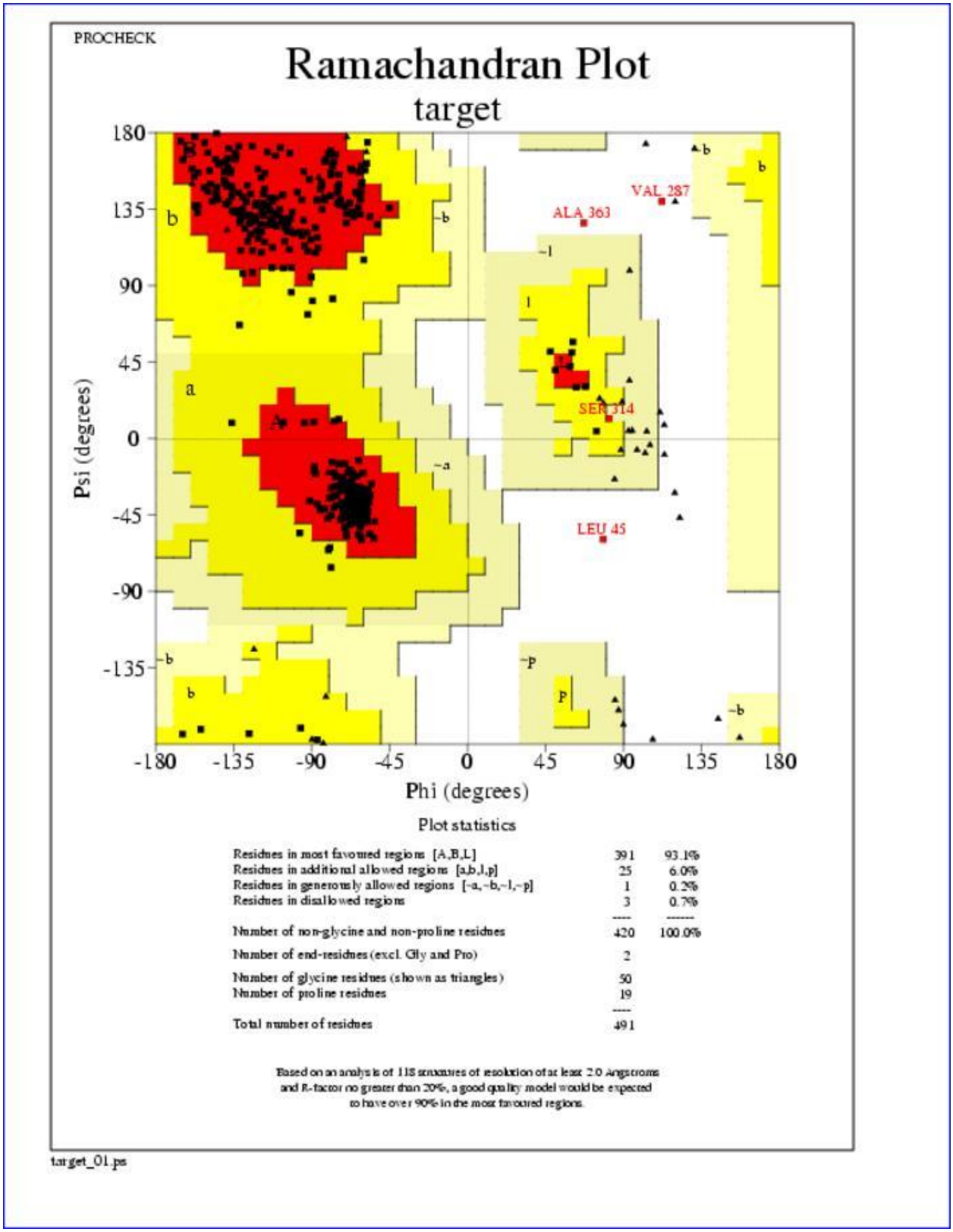


Fig 4.2: Ramachandran plot as showing the stability of the predicted model

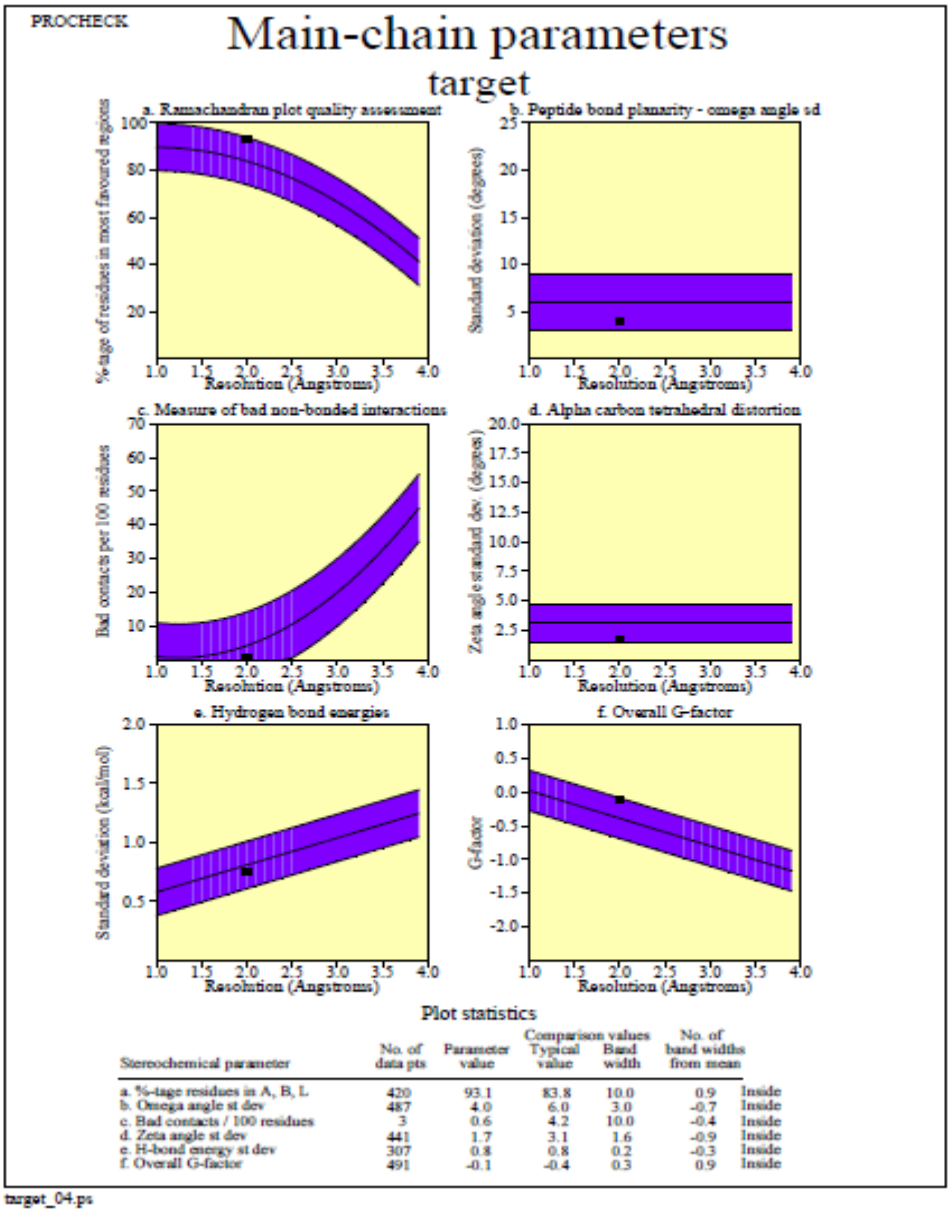


Fig 4.3: Graphs showing a. Ramachandran plot quality b. Peptide bond planarity c. Bad non-bonded interactions d. Ca tetrahedral distortion e. Main chain hydrogen bond energy f. Overall G-factor

The six graphs show how the structure black square compares with well refined structure at a similar resolution. In the above graphs the purple band in each graph represents the results from the well-refined structures; the central line is least-squares fit to the mean trend as a function of resolution and the width of the band on either side of it correspond to a variation

of one standard deviation about the mean. As the structure in every case comes inside the band this signifies the stability of the structure.

4.1.3 Identification of active site

The active site was given as HIS461 in the UNIPROT but for further clarification CASTp was used which gave the largest pocket of area 2559.3 and Volume 3950.3. There are various residues participating in the pocket.

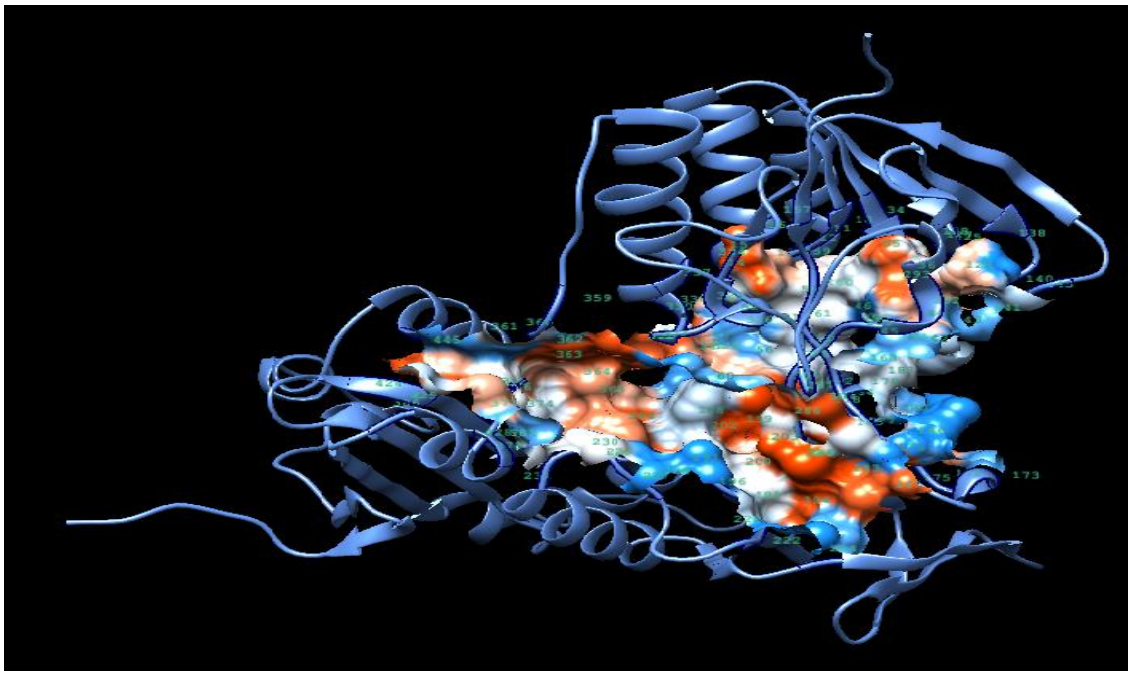


Fig 4.4: CASTp image showing the pocket 1 and the participating residues as viewed in CHIMERA

4.1.4 Superimposition results

The superimposition of the TPR and GR showed that the binding site of both the enzymes is different and one substrate binding with the TPR will not bind with GR. This interaction study was necessary to make sure that the drug designed against TPR does not interact with GR in humans causing any side effects. This was done through the sequence alignment of the protein sequence of GR from humans and TPR from *L.donovani* and later superimposing both the structures to show the difference in the secondary structure.

```
sp|P39050|TYTR_LEIDO        MSRAYDLVVLGAGSGGLEAG-------------WNAAVTHKKKVAVVDVQATHGPP----
sp|P00390|GSHR_HUMAN        MALLPRALSAGAGPSWRRAARAFRGFLLLLPEPAALTRALSRAMACRQEPQPQGPPPAAG
                            *:      :  ***..  .*.                  : : .: :*  :   .:***

sp|P39050|TYTR_LEIDO        ---------------------------------ALVALGGTCVNVGCVPKKLMVTGAQYM
sp|P00390|GSHR_HUMAN        AVASYDYLVIGGGSGGLASARRAAELGARAAVVESHKLGGTCVNVGCVPKKVMWNTAVHS
                                                            ***************:* . * :

sp|P39050|TYTR_LEIDO        DLIRESGGFGWEMDRESLCPNWKTLIAAKNKVVNSINESYKSMFADTEGLSFHMGFGALQ
sp|P00390|GSHR_HUMAN        EFMHDHADYGFPSCEG--KFNWRVIKEKRDAYVSRLNAIYQNNLTKSHIEIIRG------
                            ::::: ..:*:   .     **:.:   ::  *. :*  *:. ::.:.   ::

sp|P39050|TYTR_LEIDO        DAHTVVVRKSEDPHSDVLETLDTEYILIATG---SWPTRLGVPGDEFCITSNEAFYLEDA
sp|P00390|GSHR_HUMAN        --HAAFTSDPKPTIEVSGKKYTAPHILIATGGMPSTPHESQIPGASLGITSDGFFQLEEL
                              *:... ..: . .   :.  : :******   * * .  :** .: ***:  * **:

sp|P39050|TYTR_LEIDO        PKRMLCVGGGYIAVEFAGIFNGYKPCGGYVDLCYRGDLILRGFDTEVRKSLTKQLGANGI
sp|P00390|GSHR_HUMAN        PGRSVIVGAGYIAVEMAGILS---ALGSKTSLMIRHDKVLRSFDSMISTNCTEELENAGV
                            * * : **.******:***:.   . *. ..*  * * :**.**: : .. *::*   *:

sp|P39050|TYTR_LEIDO        RVRTNLNPTKITKNEDGSNHVHFNDG--------TEEDYDQVMLAIG-VPRSQALQLDKA
sp|P00390|GSHR_HUMAN        EVLKFSQVKEVKKTLSGLEVSMVTAVPGRLPVMTMIPDVDCLLWAIGRVPNTKDLSLNKL
                            .* .  : .::.*. .* :   ..              * * :: *** **.:: *.*:*

sp|P39050|TYTR_LEIDO        GVRTGKNGAVQVDAYSKTSVDNIYAIGDVTNRVMLTPVAINEGACVLLETVFGGKPRATD
sp|P00390|GSHR_HUMAN        GIQTDDKGHIIVDEFQNTNVKGIYAVGDVCGKALLTPVAIAAGRKLAHRLFEYKEDSKLD
                            *:::*.:* : ** :.:*.*..***:*** .:.:******  *  :  . .   :    *

sp|P39050|TYTR_LEIDO        HTKVACAVFSIPPIGTCGMTEEEAAKNY--ETVAVYASSFTPLMHNISGSKHKEFMIRII
sp|P00390|GSHR_HUMAN        YNNIPTVVFSHPPIGTVGLTEDEAIHKYGIENVKTYSTSFTPMYHAVTKRKTKCVMKMVC
                            :.::. .*** ***** *:**:** ::*  *.* .*::****: * ::  * * .*  :

sp|P39050|TYTR_LEIDO        TNESNGEVLGVHMLGDSAPEIIQSVGICMKMGAKISDFHSTIGVHPTSAEELCSMRTPAY
sp|P00390|GSHR_HUMAN        ANKEE-KVVGIHMQGLGCDEMLQGFAVAVKMGATKADFDNTVAIHPTSSEELVTLR----
                            :*:.: :*:*:** * .. *::*...:.:****. :**..*:.:****:*** ::*

sp|P39050|TYTR_LEIDO        FYESGKRVEKLSSNL
sp|P00390|GSHR_HUMAN        ---------------
```

Fig 4.5: Sequence alignment of GR of Humans and TPR of *L.donovani*

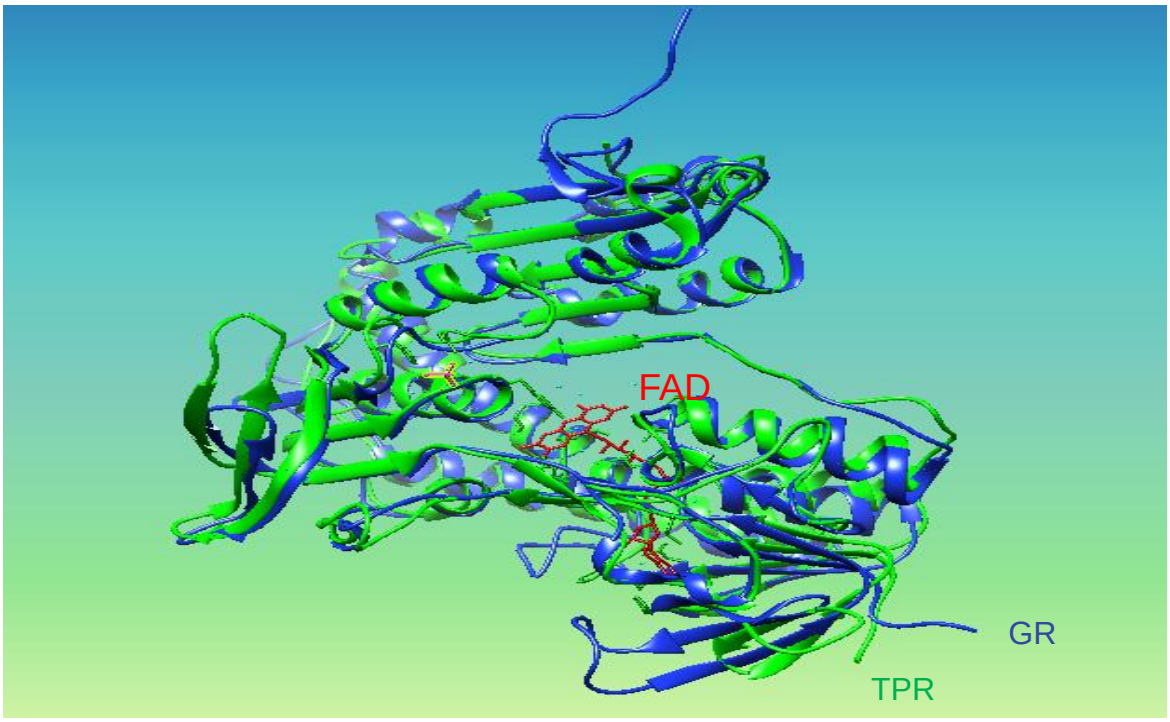


Fig 4.6: Showing superimposition of TPR and GR and their interaction with FAD

The superimposition image clearly showed that the binding site of TPR and GR is different. The LIGPLOT analysis of the FAD binding site further showed the varied interacting residues.

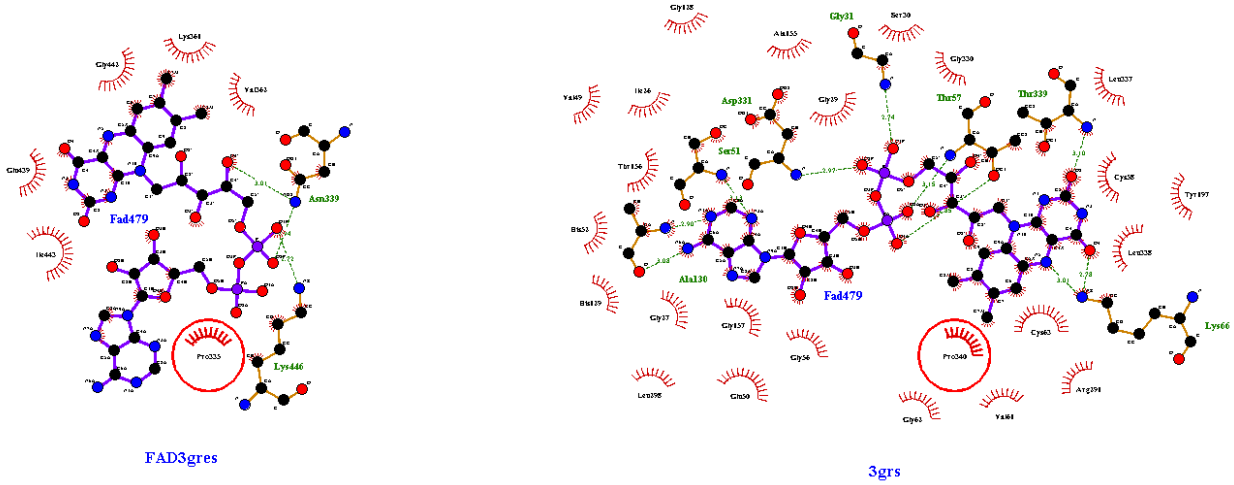


Fig 4.7: LIGPLOT analysis of TPR and GR showing the difference in their interacting residues

4.1.5 Docking

Various ligands were taken on the basis of their action on the variants of the enzyme in other species and the other strains of *Leishmania*. The 3D structure was obtained from the Pubchem and for those the 3D structures were not available online tool CORINA was used for generation of the 3D structure. The 3D structures of ligands were docked with the modeled 3D structure of the enzyme and their affectivity was seen on the basis of their binding energy. Effective results were obtained using the Autodock 4.2 tool which provided some ligands which can be taken for further study on drug discovery.

Top 50 results are shown in the table below:

S.no	Ligand	Phytochemical type	Pubchem/ CHEMSPIDER ID	Molecular formula	Molecular weight (Da)	H-bond donor	H-bond acceptor	Binding energy
1	Taxifolin	Flavononol	CID 439533	$C_{15}H_{12}O_7$	304.2518	5	7	-8.1
2	Tomatine	Alkaloid	CID 28523	$C_{50}H_{83}NO_{21}$	1034.188	13	22	-8.0

3	Curcumin	Curcuminoid	CID 969516	$C_{21}H_{20}O_6$	368.3799	2	6	-7.9
4	Biochanin A	Isoflavones	CID 5280373	$C_{16}H_{12}O_5$	284.26348	2	5	-7.9
5	Naringin	Flavonoid	CID 442428	$C_{27}H_{32}O_{14}$	580.53458	8	14	-8.0
6	Garcinia	Biflavonoid	CID 161259	$C_{30}H_{22}O_{12}$	574.48848	8	12	-7.9
7	Celastrol	Celastroid	CID 122724	$C_{29}H_{38}O_4$	450.60962	2	4	-7.9
8	Morphinan	Alkaloid	16735917	$C_{16}H_{21}N$	227.34460	1	1	-7.6
9	Quinidine	Alkaloid	CID 441074	$C_{20}H_{24}N_2O_2$	324.41676	1	4	-7.6
10	Nobiletin	Flavonoid	CID 72344	$C_{21}H_{22}O_8$	402.3945	0	8	-7.6
11	ProcyanidinB2	Proanthocyanidin	CID 122738	$C_{30}H_{26}O_{12}$	578.52024	10	12	-7.5
12	Thermopsine	Alkaloid	CID 638234	$C_{15}H_{20}N_2O$	244.3321	0	2	-7.4
13	Piperine	Alkaloid	553590	$C_{17}H_{19}NO_3$	285.3377	0	4	-7.4
14	Hesperetin	Bioflavonoid	CID 72281	$C_{16}H_{14}O_6$	302.27876	3	6	-7.4
15	Quinine	Alkaloid	CID 8549	$C_{20}H_{24}N_2O_2$	324.41676	1	4	-7.4
16	Kaempferol	Flavonol	CID 5280863	$C_{15}H_{10}O_6$	286.2363	4	6	-7.4
17	Rhein	Anthraquinone	CID 10168	$C_{15}H_8O_6$	284.22042	3	6	-7.3
18	Demethylnobiletin	Flavonoid	CID 358832	$C_{20}H_{20}O_8$	388.368	1	8	-7.3
19	Rhombinin	Flavonoid	CID 228640	$C_{15}H_{20}N_2O$	244.3321	0	2	-7.2
20	Salsoline	Alkaloid	CID 46695	$C_{11}H_{15}NO_2$	193.2423	2	3	-7.2
21	Eicosane	Alkaloid	CID 8222	$C_{20}H_{42}$	282.54748	0	0	-7.2
22	Naringenin	Flavonoid	CID 932	$C_{15}H_{12}O_5$	272.25278	3	5	-7.2
23	Chrysophanic acid	Flavonoid	CID 10208	$C_{15}H_{10}O_4$	254.2375	2	4	-7.2
24	Oxyanthraquinone	Flavonoid	CID 53433123	$C_{14}H_8O_3$	224.21152	0	3	-7.2
25	Salsolidine	Alkaloid	CID 10302	$C_{12}H_{17}NO_2$	207.26888	1	3	-7.1
26	Colchicine	Alkaloid	CID 6167	$C_{22}H_{25}NO_6$	399.437	1	6	-7.1
27	Emetin	Alkaloid	CID 10219	$C_{29}H_{40}N_2O_4$	480.6389	1	6	-7.1

28	Brazilein	Celastroid	CID 6453902	$C_{16}H_{12}O_5$	284.26348	3	5	-7.1
29	Duartin	Flavonoid	CID 3666064	$C_{18}H_{20}O_6$	332.3478	2	6	-7.1
30	Emodin	Celastroid	CID 3220	$C_{15}H_{10}O_5$	270.2369	3	5	-7.1
31	Epiafzelechin	Flavanol	CID 443639	$C_{15}H_{14}O_5$	274.26866	4	5	-7.1
32	Epicatechin	Flavonoid	CID 72276	$C_{15}H_{14}O_6$	290.26806	5	6	-7.1
33	Fistucacidin	Flavonoid	4476230	$C_{15}H_{14}O_6$	290.268097	5	6	-7.1
34	Leucopelargonidin	Flavonoid	CID 3286789	$C_{15}H_{14}O_6$	290.2680	5	6	-7.1
35	Lupinine	Alkaloid	CID 91461	$C_{10}H_{19}NO$	169.26396	1	2	-7.0
36	Asphyllic acid	Flavonoid	CID 171568	$C_{15}H_{22}O_2$	234.33398	1	2	-7.0
37	Coralyne chloride	Alkaloid	CID 23306	$C_{22}H_{22}ClNO_4$	399.86738	0	5	-7.0
38	Cosmosin	Flavonoid	CID 23663965	$C_{16}H_{14}N_7NaO_5S_4$	535.576029	2	14	-7.0
39	Quercetin	Flavonol	CID 5280343	$C_{15}H_{10}O_7$	302.2357	5	7	-7.0
40	Catechin	Flavanol	CID 73160	$C_{15}H_{14}O_6$	290.26806	5	6	-7.0
41	Iridin	Flavonoid	4445090	$C_{24}H_{26}O_{13}$	522.455383	6	13	-6.9
42	Tangeritin	Flavonoid	CID 68077	$C_{20}H_{20}O_7$	372.3686	0	7	-6.7
43	Daidzein	Isoflavone	CID 5281708	$C_{15}H_{10}O_4$	254.2375	2	4	-6.7
44	Palmatine chloride	Alkaloid	CID 73442	$C_{21}H_{22}ClNO_4$	387.8566	0	5	-6.6
45	Genistin	Flavonoid	CID 5281377	$C_{21}H_{20}O_{10}$	432.3775	6	10	-6.6
46	Heptacosane	Alkaloid	CID 11636	$C_{27}H_{56}$	380.73354	0	0	-6.6
47	Formononetin	Flavonoid	CID 5280378	$C_{16}H_{12}O_4$	268.26408	1	4	-6.5
48	Cytisine	Alkaloid	CID 10235	$C_{11}H_{14}N_2O$	190.24166	1	2	-6.4
49	Octacosane	Alkaloid	CID 12408	$C_{28}H_{58}$	394.76012	0	0	-6.4
50	Gardenin B	Flavonoid	CID 96539	$C_{19}H_{18}O_7$	358.34202	1	7	-6.4

Table 4.1: Top 50 ligands showing high affinity to TPR

Instead of showing higher binding affinity Taxifolin, Tomatine and Naringin break the Lipinski's rule of 5 which is one of the most prerequisite condition for the selection of any compound as chemotherapeutic agent. The next best phytochemical Curcumin is a curcuminoid which is obtained from the roots of Curcuma longa. Curcumin clearly shows high affinity towards TPR and its binding energy. The Ligplot analysis of Curcumin as shown:

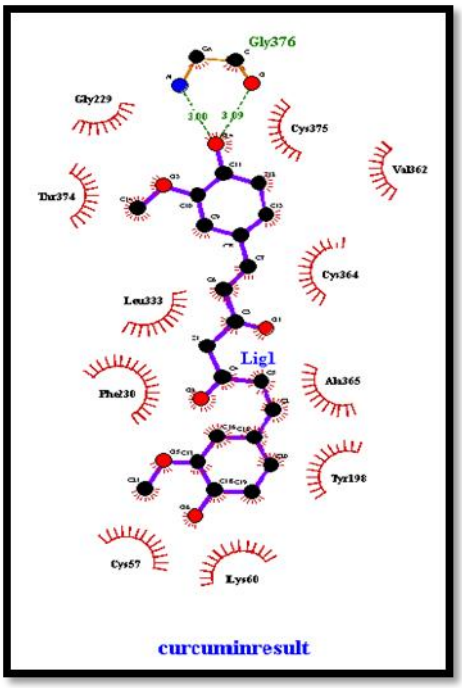
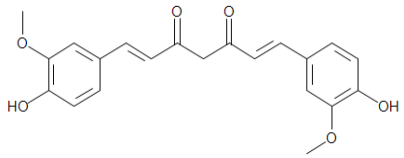
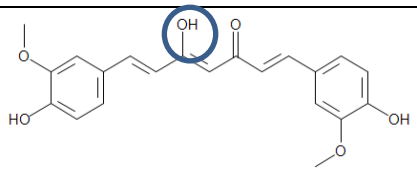
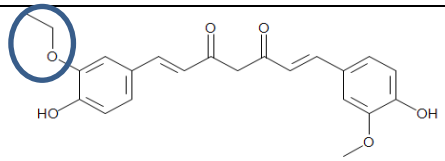
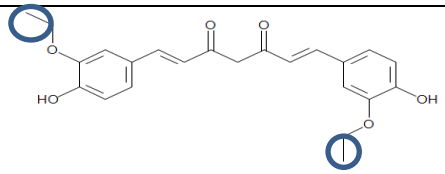
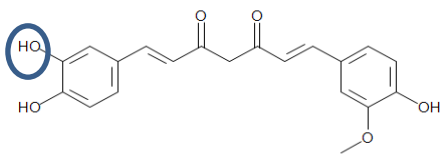
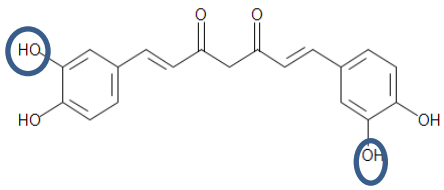
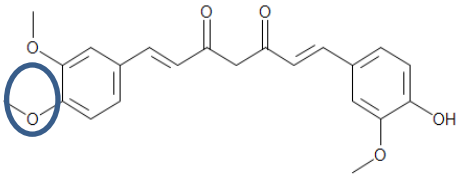
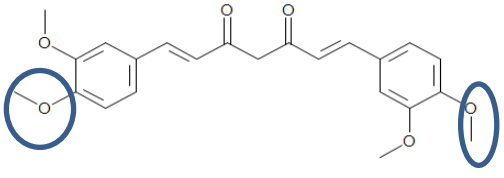


Fig 4.8: Ligplot analysis of Curcumin and TPR

The Ligplot analysis clearly shows the hydrophobic residues involved in the interaction with Curcumin are Gly229, Thr374, Leu333, Phe230, Cys57, Lys60, Tyr198, Ala365, Cys364, Val362, Cys375, Gly376. The hydrophobic residue Gly376 is involved in the hydrogen bonding. Even though curcumin showed higher affinity, designing of novel compounds taking curcumin as the base chain can provide better binding affinity and can be synthesized to overcome the low drug score and low availability of Curcumin.

4.1.6 Design of the Analogues

The analogues were designed using MEDCHEM DESIGNER, keeping the compound ligand constant and varying the side chains. 25 analogues were designed for the further study. The top 13 ligands which showed better binding affinity than the original ligand are shown in the table:

S.no	Structure	Molecular formula	Binding energy
1		Original	-7.9
2		$C_{21}H_{20}O_6$	-8.3
3		$C_{22}H_{22}O_6$	-8.6
4		$C_{23}H_{24}O_6$	-8.4
5		$C_{20}H_{18}O_6$	-8.5
6		$C_{19}H_{16}O_6$	-8.7
7		$C_{22}H_{22}O_6$	-8.4
8		$C_{23}H_{24}O_6$	-8.5

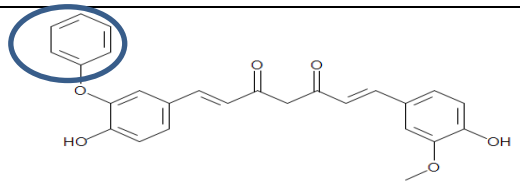
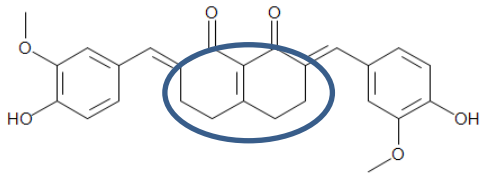
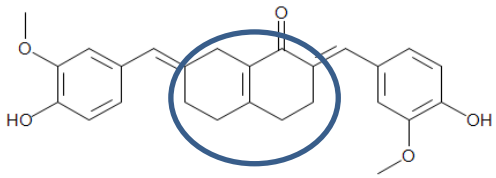
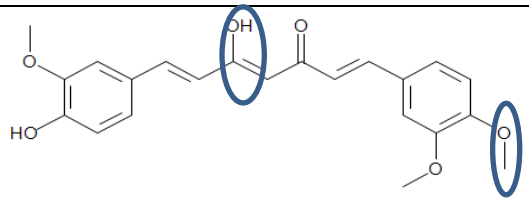
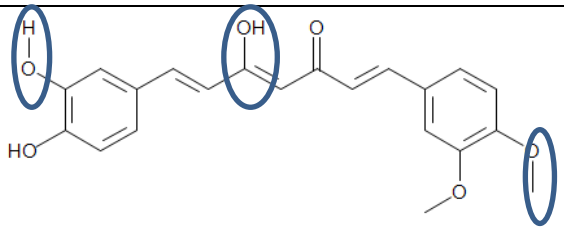
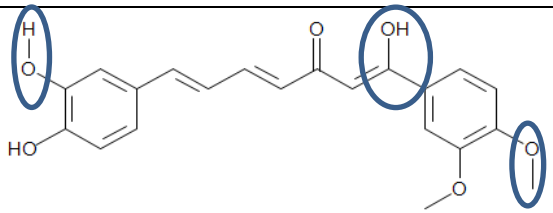
9		$C_{26}H_{22}O_6$	**-8.9**
10		$C_{26}H_{24}O_6$	**-11.1**
11		$C_{26}H_{26}O_5$	**-11.0**
12		$C_{22}H_{22}O_6$	**-11.0**
13		$C_{21}H_{20}O_6$	**-8.8**
14		$C_{21}H_{20}O_6$	-8.7

Table 4.2 Binding energy of top 13 analogues

Analogue 9,10, 11, 12 and 13 showed highest binding energy, increasing the affinity of the ligand to the enzyme manifolds which signified the better protein-ligand interaction which plays role in further inhibition of TPR.

4.1.7 ADME/T Test

The ADME/T test of the resultant analogues was done through OSIRIS and MOLSOFT. This provided the insight on the drug likeness of the ligands, which is necessary to consider before designing of a drug. To use a compound as chemotherapeutic agent it should first clear the toxicity test. The table shows druglikeness score of the top 13 analogues. These are the analogues which don't show any mutagenic, tumorogenic, irritant and reproductive effective. For instance one of the analogues showed tumorogenic property which reduces its liability as chemotherapeutic agent and thus cannot be considered for further purpose.

S.no	clogP	Solubility	MW	TPSA	Drug likeness	Drug Score	HBA	HBD
1	2.95	-3.62	368	93.06	-2.83	0.41	6	2
2	3.58	-3.67	368	96.22	1.91	0.69	6	3
3	3.36	-3.92	382	93.06	-6.44	0.36	6	2
4	3.76	-4.22	396	93.06	-6.67	0.33	6	2
5	2.67	-3.31	354	104	-3.27	0.42	6	3
6	2.4	-2.99	340	115	-3.48	0.44	6	4
7	3.22	-3.94	382	82.06	-1.99	0.41	6	1
8	3.5	-4.25	396	71.06	-2.95	0.37	6	0
9	4.41	-5.9	430	93.06	-4.87	0.23	6	2
10	4.29	-4.33	432	93.06	0.8	0.5	6	2
11	5.0	-4.68	418	75.99	0.63	0.42	5	2
12	**3.86**	**-3.99**	**382**	**85.22**	**3.99**	**0.68**	**6**	**2**
13	**3.58**	**-3.67**	**368**	**96.22**	**2.99**	**0.72**	**6**	**3**
14	3.58	-3.67	368	96.22	4.54	0.74	6	3

Table 4.3: ADME/T results for curcumin and 13 analogues as obtained from OSIRIS server (red mark shows violation of the parameter)

clogP: compound's hydrophilicity, must not be greater than 5.0

solubility: signifies absorption and distribution of drug, must be greater than -4.0

MW: Molecular Weight, less than 450

TPSA: The Polar Surface Area Prediction

Drug likeness: in the range of greater than -1.0 and less than 5

Drug score: Efficient in the range of 0 to 2

HBA: Hydrogen Bond Acceptor (not more than10)

HBD: Hydrogen Bond Donor (not more than 5)

The ADME/T test provided the drug likeness and drug score of the analogues. This sorted out the analogues which can be used for further drug discovery. Out of the 5 above analogues analogue 12 and 13 qualified as effective chemotherapeutic agent. These analogues not only showed better binding affinity but also showed minimum toxicity level and higher drug score. The further toxicity of these 2 analogues was done using DruLiTo software which provides insight to the qualification of drugs according to various rules for filtering of drug like compounds.

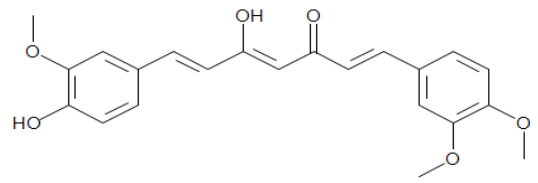
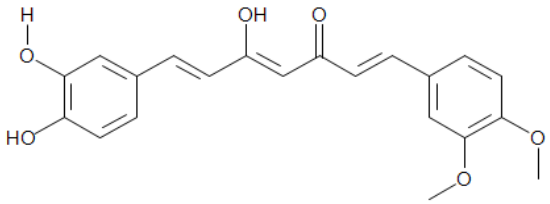
S.no	Analogue	Rules qualified
1		Lipinski's rule of 5, Ghose filter, Veber filter, Unweighted QED, Weighted QED
2		Lipinski's rule of 5, Ghose filter, Veber filter, Unweighted QED, Weighted QED

Table 4.4:Rules qualified by the specified analogues

Lipinski's rule of 5

- Less than 5 hydrogen bond donors
- Less than 10 hydrogen bond acceptors
- Molecular mass less than 500 daltons
- logP less than 5

Ghose Filter

- logP –0.4 ~5.6
- Molecular mass 160 ~ 480
- Polar surface area < 140
- Molar refractivity 40 ~ 130
- Number of atoms 20 ~ 70

Veber filter

- PSA ≤ 140
- Rotatable bond count ≤ 10

QED (Quantitative Estimation of Drug-likeness)

- Drug likeness -1.0 ~ 5.0

4.1.8 Docked image and Ligplot analysis

Docked images show the interaction of the analogues and its binding with the enzyme. The docking image was viewed using CHIMERA. Ligplot analysis is done to observe the interacting residues between the compound and the enzyme. The Ligplot of the Analogue 12 and 13 are given below:

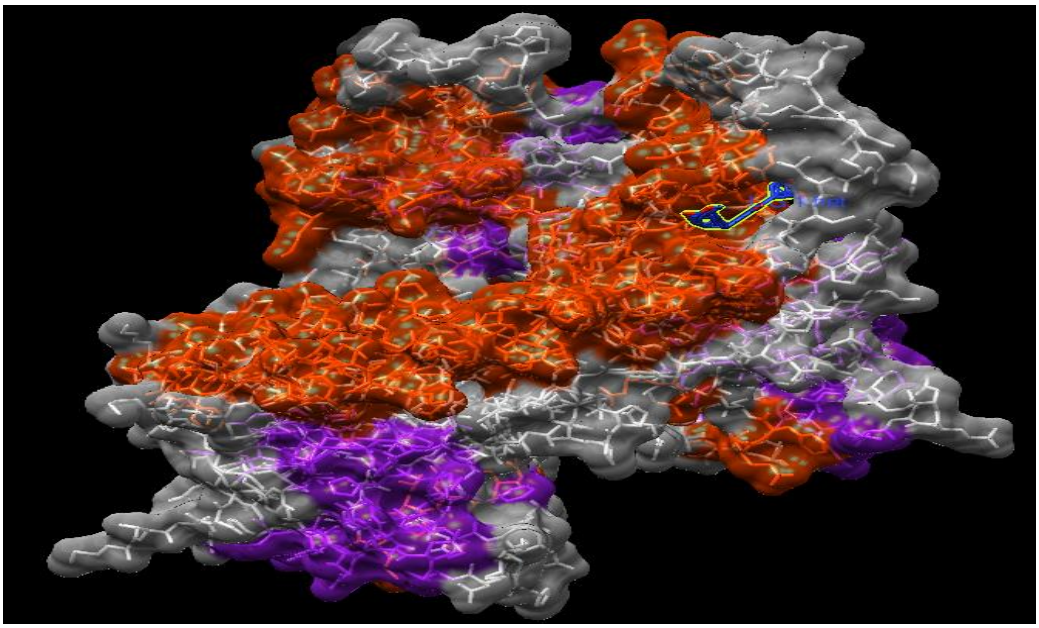


Fig 4.9: Interaction of Analogue 12 and Enzyme as obtained by Autodock 4.2 observed in Chimera

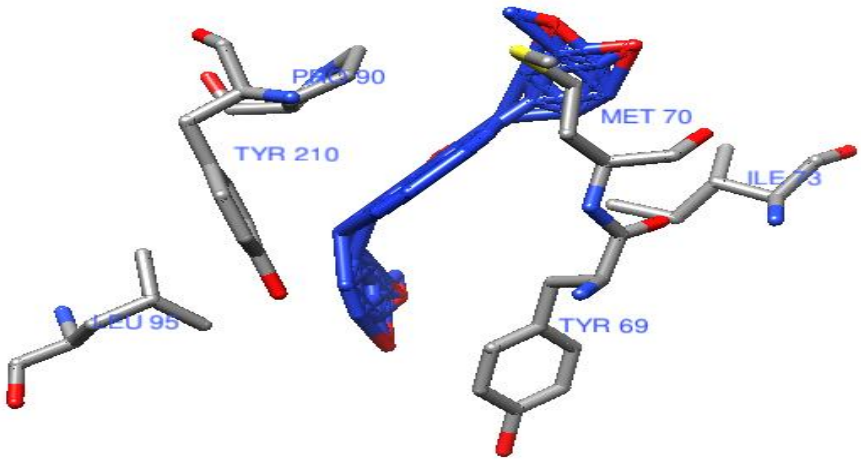


Fig 4.10: Residues of the enzyme interacting with the analogue 12 visualized in Chimera

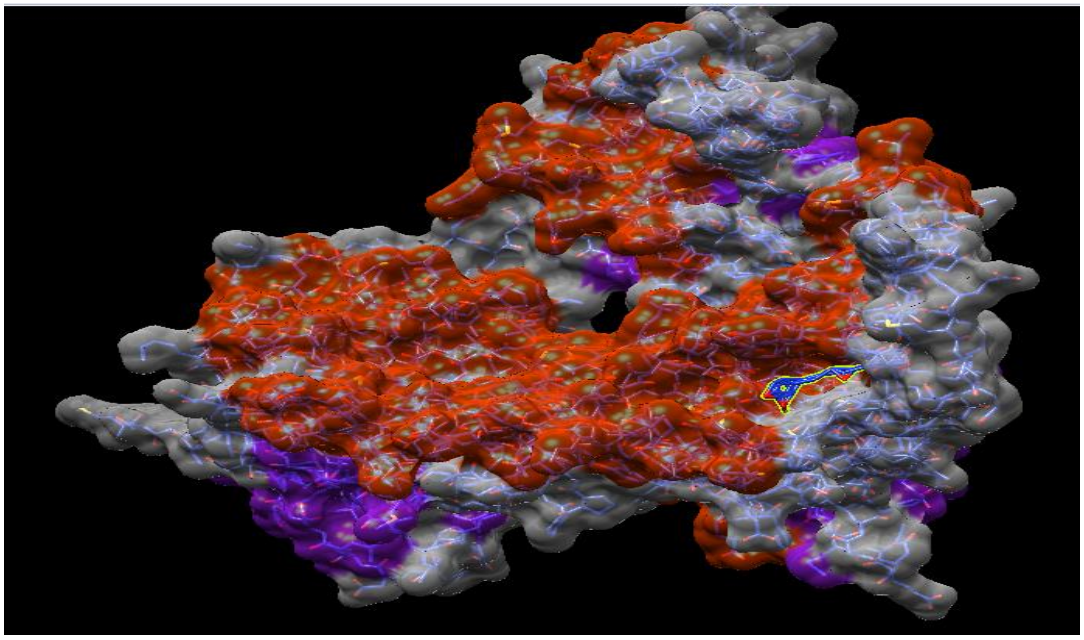


Fig 4.11: Interaction of Analogue 13 and TPR as obtained by Autodock 4.2 visualized in Chimera

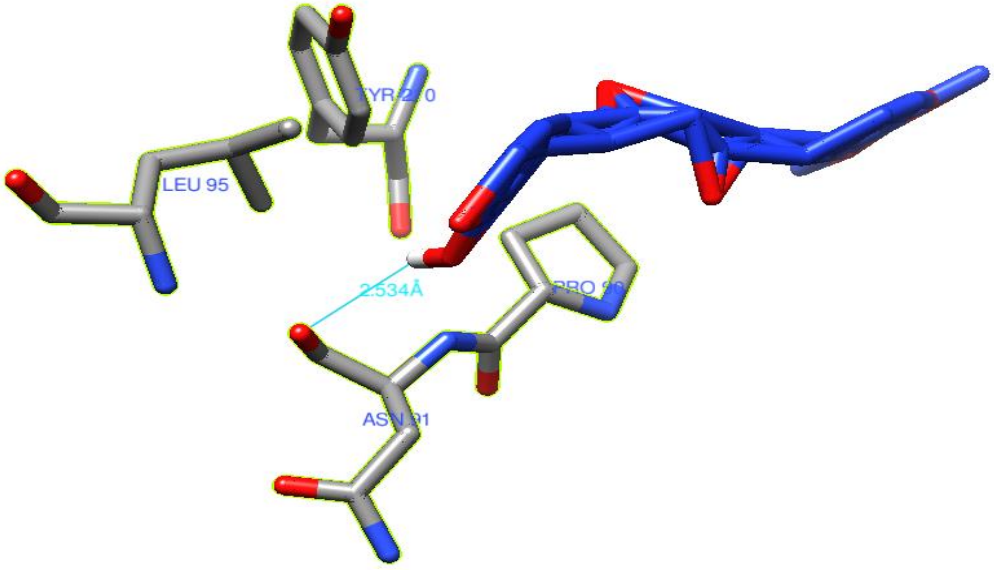


Fig 4.12: Residues of TPR interacting with analogue 13 visualized in Chimera

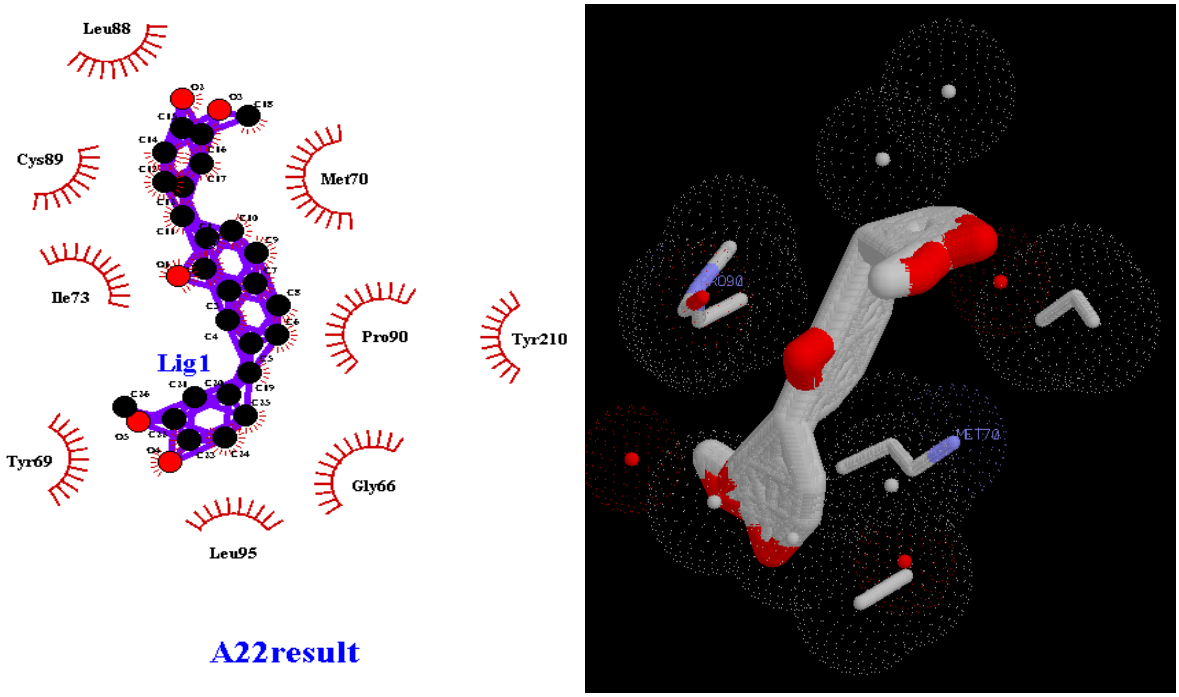


Fig 4.13: Ligplot analysis of Analogue 12 as viewed in LIGPLOT+ and RASMOL

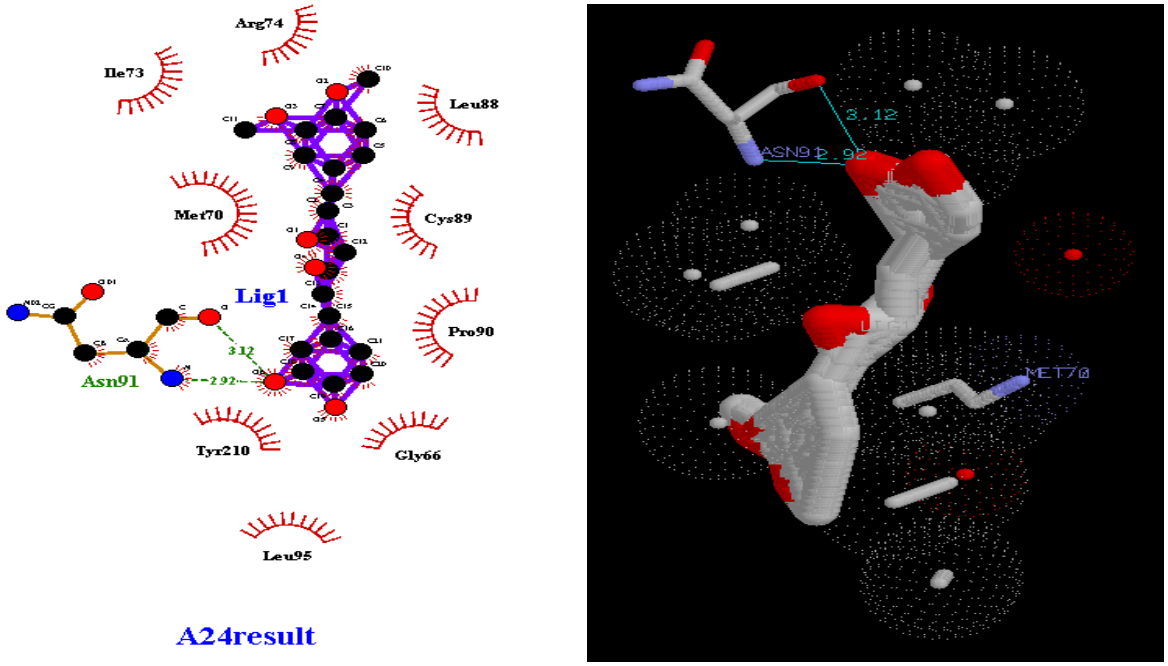


Fig 4.14: Ligplot analysis of Analogue 13 as viewed in LIGPLOT+ and RASMOL

The docked images as seen in CHIMERA shows the interaction of the ligand with nearby residues. Ligand binds to the specified binding site which displays the protein-ligand interaction and results in the formation of more stable complex.

LIGPLOT shows the 2D interaction of the ligand and the hydrophobic residues of the protein. The interacting hydrophobic residues for analogue 12 are: Leu88, Cys89, Ile73, Tyr69, Leu95, Gly66, Tyr210, Pro90 andMet70.

The interacting hydrophobic residues for analogue 13 are: Arg74, Ile73, Met70, Cys89, Pro90, Leu88, Gly66, Leu95, Tyr210 and it forms hydrogen bond with Asn91.

4.2 Discussion

3D structure of TPR in *L.donovani* have been created and validated providing a efficient drug target in Anti-Leishmanial Drug discovery. The validated structure showed high structural stability. The modeled structure showed high ERRAT score of 90.24% which displays its stability. The superimposition of TPR and GR showed the difference in active binding site. Over 500 ligands and phytochemicals were searched and the top binding affinity obtained by Taxifolin (-8.1 Kcal/mol) but Curcumin (-7.9 Kcal/mol) was taken for further purpose because of its various pharmacological purpose. Out of 25 designed analogues 13 analogues were taken on account of there binding energy. Top 5 analogues were- Analogue 9, 10 , 11, 12 and 13 showing higher binding energy as -8.9 kcal/mol, -11.1 kcal/mol, -11.0 kcal/mol, -11.0 kcal/mol and -8.8 kcal/mol. No analogue showed any toxicity and were quite feasible. However, analogue 9, 10 and 11 were having the higher logS value which decreases their compatibility as drugs. Clearly analogue 12 and 13 with a drug score of 0.68 and 0.72 were the best analogues to be taken for further drug discovery.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTIVES

5.1 Conclusion

The structure designing of enzymes implies the onset of Structure based Drug designing by finding out effective lead compounds which can result in inhibition of the enzyme in the protozoan.As the enzyme is specific to the parasite it indicates targeted drug action. Leishmaniasis is one of the neglected tropical diseases which imposes quite a threat. Various efforts to find out effective drug for its tratement have proved not to be efficient. Thus, if by any way an effective ligand can be found for inhibition of the growth of the protozoan it will be a breakthrough in researches being carried for the purpose. The ligands which were thus found to be effective as inhibitor for the target protein are mainly the phytochemicals. The ligand Curcumin give quite decent binding energy result and this can be used for further study of drug designing. This study thus enlighten one of the hidden aspect of drug designing for Leishmaniasis which is one of the delinquent part right now and thus can facilitate further drug designing process. Efficacy of Phytochemicals to be used as drugs is based on their broad spectrum of action, easy availability and low toxicity. Phytochemicals are being used efficiently for drug designing. For Leishmaniasis too the phytochemicals can be used for drug designing and the risk of present therapeutics can be avoided.

5.2 Future perspective

Structure based drug designing and Lead discovery have been used efficiently for drug designing. The results obtained by the present work is based on accurate and reliable softwares which authenticates their usability. The initial prospect of In silico studies prevent a lot of cost and work of in vitro and in vivo experiments. As novel compounds designing has been done and it has shown remarkable results,it can be used for further Molecular Dynamics Simulation to generate the real time behaviour system. Further the result can be corroborated by in-vitro and in-vivo studies which will not only lead to new prospect of drug designing but also provide us with effective Anti-leishmanial therapeutics.

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